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▲ To whom inquiries should be directed.

Kinetics and Mechanisms of Drug Action on Microorganisms XVII: Bactericidal Effects of Penicillin, Kanamycin, and Rifampin with and without Organism Pretreatment with Bacteriostatic Chloramphenicol, Tetracycline, and Novobiocin

EDWARD R. GARRETT▲ and CHONG MIN WON

Abstract □ *Escherichia coli* (ATCC 12407), generating in the logarithmic growth phase, was killed by penicillin, kanamycin, and rifampin. The logarithm of number of viables of the drug-treated culture decreased linearly with time after a certain lag period and above a minimum drug concentration. The slopes of these plots were characteristic of kill rate constants and were linearly dependent on drug concentrations. The microorganisms developed resistance against the cidal action of the antibiotics. The lower the penicillin concentration and the rate of kill, the greater is the number of resistant organisms, most probably formed by generation in the presence of penicillin. Penicillin was inactivated with time in the culture of organisms, and the resistant individuals eventually grew and were shown to be insensitive to penicillin. When organisms were exposed to the cidal action of kanamycin or rifampin, the appearance of resistant individuals was ascertained. Penicillin yielded debris and ghost cells of *E. coli*. No such apparent lysis was observed with kanamycin or rifampin. The activity of kanamycin increased with the pH of the medium, so activity can be assigned to the uncharged or lesser charged fraction of the drug concentration. The addition of penicillin or kanamycin to the organisms treated with tetracycline, chloramphenicol, or novobiocin did not show any significant difference in killing rate from organisms not previously treated with such bacteriostatic agents. However, the combination of a bactericidal and a bacteriostatic antibiotic depressed the resistant mutant formation of resistant individuals over that of the bactericidal drug alone. When rifampin was added to organisms pretreated with tetracycline or chloramphenicol, the cidal action of rifampin was significantly reduced.

Keyphrases □ Bactericidal activity, kinetics and mechanisms—penicillin, kanamycin, and rifampin with and without *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin □ Antibiotics, kinetics and mechanisms of bactericidal activity—penicillin, kanamycin, and rifampin with and without *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin □ Bacteriostatic *E. coli* pretreatment with chloramphenicol, tetracycline, or novobiocin—effect on bactericidal activity of penicillin, kanamycin, and rifampin

Antibacterial agents have two possible modes of action: inhibition of microbial generation and/or "kill" superimposed on generation. They can be differentiated by concomitantly monitoring the microorganism concentration of the nutrient medium containing the agent by viable (colony) counts and total (Coulter) counts as a function of time. It was shown (1) that the total counts and colony counts are coincident for the bacteriostatic tetracyclines, chloramphenicol, sulfonamides, macrolides, lincosaminides, *etc.*, in the concentration ranges below the amount resulting in complete inhibition of microbial generation.

The bactericidal action of drugs in defined concentration ranges must be kinetically defined by the time-consuming and laborious colony count method. This article reports on studies designed to quantify systematically the effects on microbial generation of several bactericidal antibiotics: penicillin, kanamycin, and rifampin.

The primary action of penicillin is in the inhibition of the production of cell wall material, specifically in the biosynthesis of murein (2). Presumably by interacting with the acceptor site of the ribosome, kanamycin may cause significant conformational changes in ribosomes which induce ambiguity in the readout of mRNA. (2). Rifampin was shown (2) to inhibit DNA-dependent RNA synthesis by specifically interfering with the function of the RNA polymerase by forming a stable rifampin–polymerase complex.

EXPERIMENTAL

Microorganism—Replicate slants of *Escherichia coli* (ATCC 12407) were used in all experiments.

Culture Media—Bacto Antibiotic Medium 3[1], rehydrated (pH 7.05) according to the specifications of the manufacturer to Medium 3 USP, was used. The culture medium was filtered through a 0.45-μ filter[2] and autoclaved at 120° for 15 min. To obtain media in the pH range of 6.30–7.55, various amounts of concentrated hydrochloric acid and sodium hydroxide solution were added aseptically to the culture media.

Antibiotic—The assayed samples of sodium penicillin G[3] (1625

[1] Difco Laboratories Detroit, Mich.
[2] Millipore HA.
[3] Obtained from The Upjohn Co., Kalamazoo, Mich.

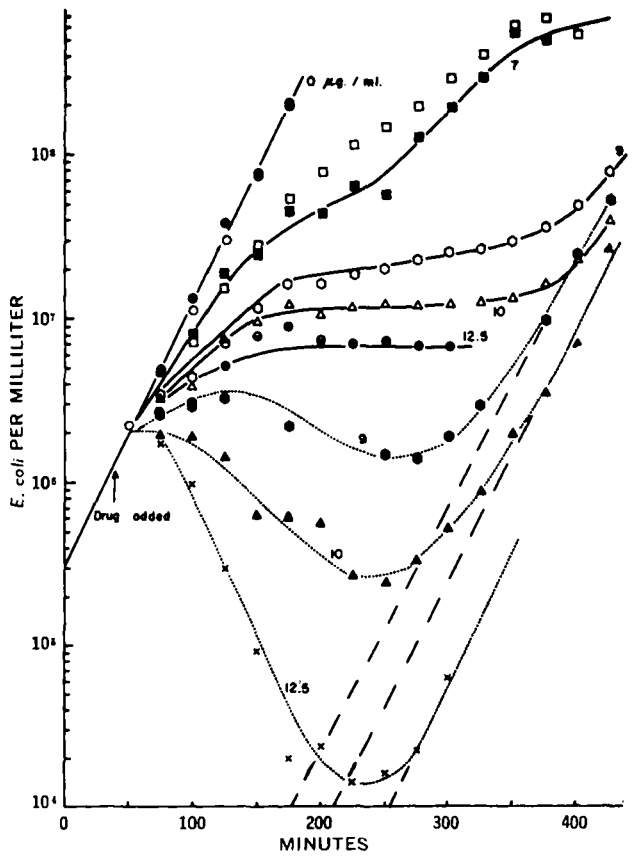


Figure 1—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of sodium penicillin G, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the sodium penicillin G concentration in micrograms per milliliter. The short dashed lines represent the logarithmic growth of the emergent resistant organisms.*

units/mg.), kanamycin sulfate[4] (795 mcg./mg.), and rifampin[5] (981 mcg./ml.) were used.

Bacterial Culture—An aliquot (5 ml.) of culture medium was inoculated from a fresh slant, and the culture was allowed to generate for 15 hr. at 37.5° in an incubator. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium. The generation of the culture was followed up to 2×10^7 *E. coli*/ml. An aliquot of this culture was added to bulk broth medium so that the organism population was diluted 100-fold. The inoculated bulk solution was shaken and kept in an incubator at 37.5° for 15 min. Aliquots (49.5 ml.) of the inoculated broth medium were then aseptically transferred into replicate, 125-ml., loosely capped conical flasks through a delivery head. The temperature of the cultures was maintained at 37.5 ± 0.01° in a constant-temperature water bath equipped with a shaker.

Total Count Method—Aliquots (1.00 or 0.50 ml.) of cultures were withdrawn at 25-min. intervals and added to an appropriate amount of diluent. The diluent was a 0.45-µ filtered[2] aqueous solution of 0.85% sodium chloride and 1% formaldehyde. This number of organisms in a 50-µl. sample was counted on the Coulter counter[6] equipped with a 30-µ orifice. The total counts were corrected for the background counts of the particular batch of medium used, which was diluted in the same way as the sample. The coincidence corrections were made by a program on a calculator[7] in accordance with the coincidence correction chart supplied by the manufacturer's manual for the Coulter counter.

Viable Count Method—Aliquots (0.50 ml.) of the drug-affected culture were withdrawn and appropriately diluted into sterilized 0.85% saline solution according to a preplanned dilution scheme. Aliquots (1.00 ml.) from those dilutions were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5°, and colonies were counted on a colony counter[8].

Effect of Bactericidal Antibiotics on Generation Rates—Fresh solutions of sodium penicillin G, kanamycin sulfate, and rifampin were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 0.50 ml. added to 49.5 ml. culture yielded the desired graded drug concentrations. The antibiotic solutions were added to the cultures generating at 37.5° in the logarithmic phase at predetermined organism populations. Samples were withdrawn every 25 min. and counted by the total count method as well as by the viable method. One culture without drug was studied in each experiment as a control. The plots of the logarithm of the total and viable numbers of *E. coli* per milliliter against time yielded generation curves for penicillin-, kanamycin-, and rifampin-affected cultures (Figs. 1–3).

Irreversibility of Kanamycin Action—Aliquots (5.0 and 0.5 ml.) of a drug-free culture generating in the logarithmic phase with an inoculum size of 1.7×10^7 *E. coli*/ml. (curve A in Fig. 4) were added to 45.0 and 49.5 ml. of fresh broth so that the organism population was diluted 10-fold (curve B) and 100-fold (curve C), respectively. A 49.5-ml. volume of broth containing 2.2×10^6 *E. coli*/ml. in the logarithmic generation phase was treated with 0.5 ml. of kanamycin sulfate solution to achieve a final concentration of 5 mcg./ml. (curve D). Twenty-five minutes after the drug addition, aliquots of curve D culture were diluted 10-fold into broth containing enough kanamycin sulfate so that the drug concentrations were adjusted to 1.7 (curve E), 2.2, and 3.3 mcg./ml. At 205 min., after the total counts of the culture of curve D reached a plateau of 1.5×10^7 *E. coli*/ml., aliquots of 5.0 and 0.5 ml. were added to 45.0 and 49.5 ml. fresh broth so that both organism and drug concentrations were diluted 10-fold (curve E) and 100-fold (curve F), respectively. At the same time, aliquots of the culture of curve D were diluted 10-fold (curve E) and 100-fold (curve F) in broths containing enough kanamycin sulfate so that the drug concentration was restored to 5 mcg./ml.

Cell Size Analyses—The size distribution of cells of *E. coli* in the

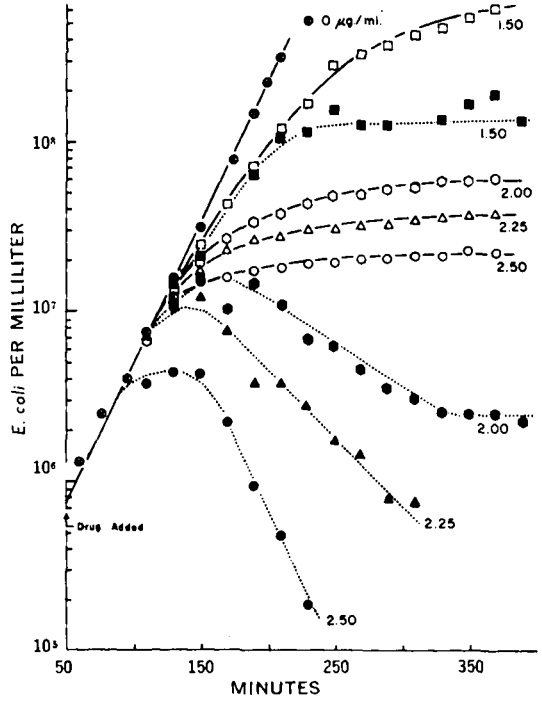


Figure 2—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of kanamycin sulfate, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the kanamycin sulfate concentration in micrograms per milliliter.*

[4] Obtained from Bristol Laboratories, Syracuse, N. Y.
[5] Obtained from Ciba-Geigy Corp., Summit, N. J.
[6] Model ZBI, Coulter Electronics, Inc., Hialeah, Fla.
[7] Wang 700.
[8] Model C-110, New Brunswick Scientific Co., New Brunswick, N. J.

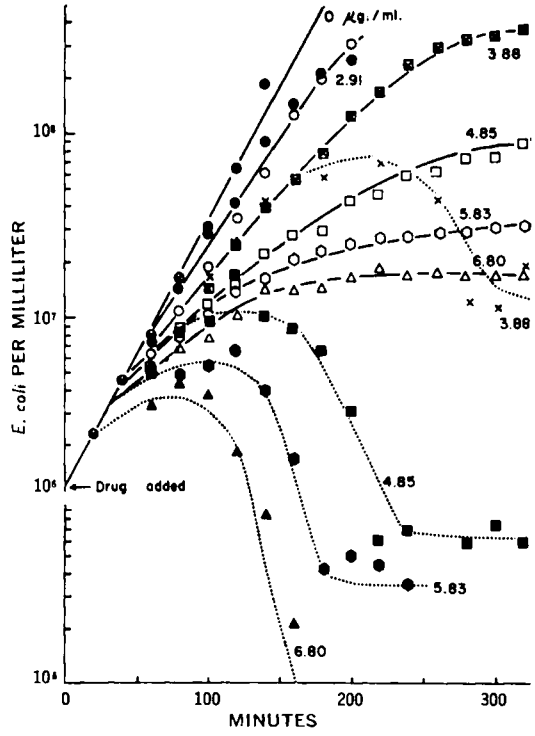


Figure 3—*Typical generation curves of* E. coli *in Medium 3 USP at pH 7.05 and 37.5° in the absence and presence of various concentrations of rifampin, obtained by total (solid lines) and viable (dashed lines) counts. The curves are labeled with the rifampin concentration in micrograms per milliliter.*

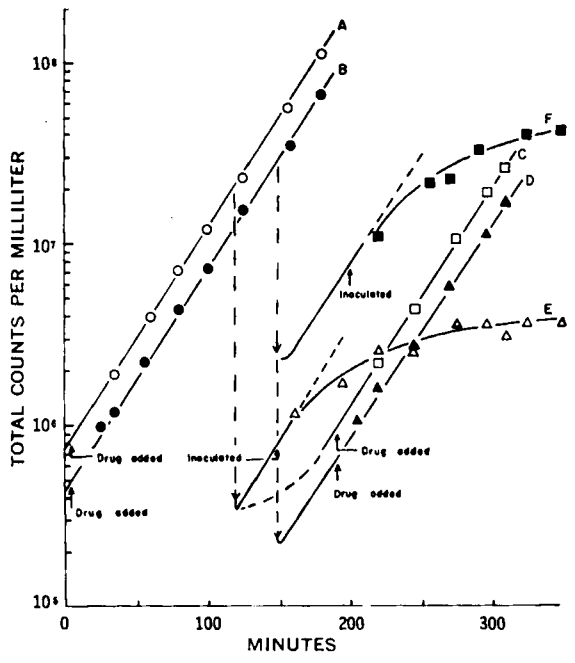


Figure 5—*Proof of development of rifampin- and kanamycin-resistant* E. coli. *Curves A and B are the generation curves for the cultures of rifampin- and kanamycin-resistant* E. coli *treated with 8.7 and 4.0 mcg./ml. rifampin and kanamycin, respectively. Curves C and D result when the filtered organisms of the cultures of curves A and B, in fresh medium, are treated with 8.7 and 4.0 mcg./ml. rifampin and kanamycin, respectively. Curves E and F result when the filtrates of the cultures of curves A and B, respectively, are inoculated with fresh* E. coli.

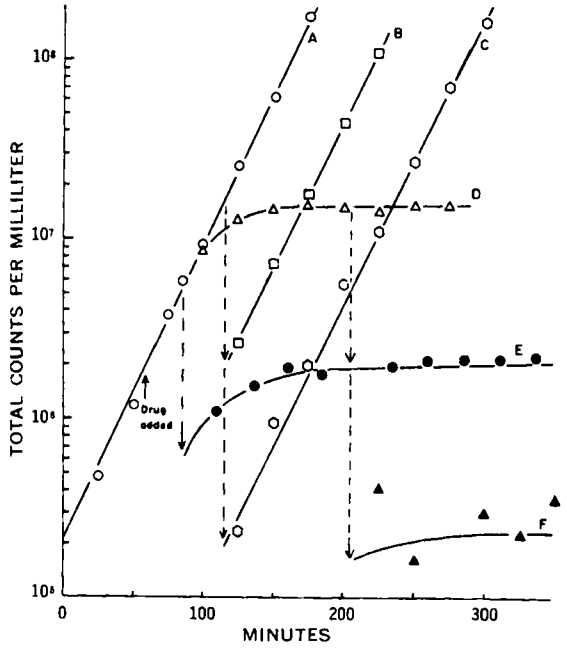


Figure 4—*Irreversibility of kanamycin sulfate-inhibited generation of* E. coli. *Curve A is for the drug-free culture. Curves B and C result after 1:10 and 1:100 dilutions, respectively, of the curve A culture with fresh broth. Curve D results after the addition of kanamycin sulfate to achieve a final concentration of 5 mcg./ml. Curve E results after the 1:10 dilution of curve D at 25 min. with broth containing kanamycin sulfate at a final concentration of 1.7 mcg./ml. Curve E also results after 1:10 dilution of curve D at 205 min. with final concentrations of 0.5 and 5 mcg./ml. of kanamycin sulfate. Curve F results after 1:100 dilution of curve D and with final concentrations of 0.05 and 5 mcg./ml. of kanamycin sulfate.*

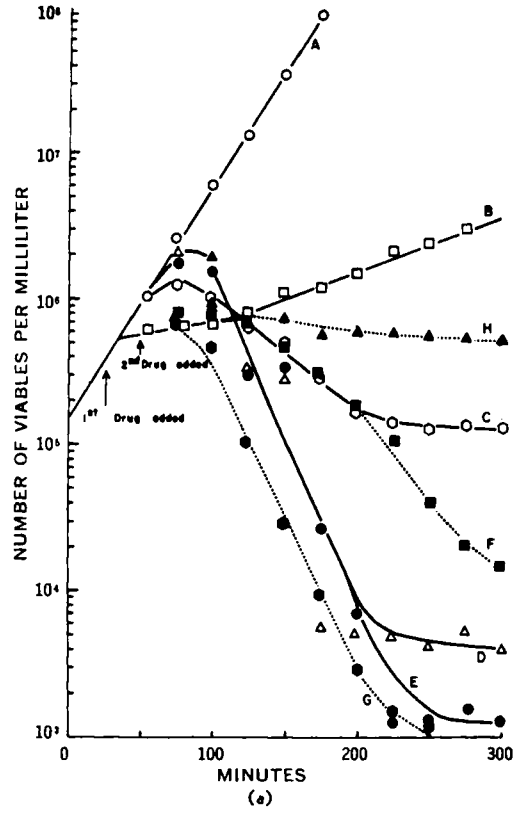
presence or absence of penicillin, kanamycin, and rifampin was studied using the Coulter counter in conjunction with a Channelyzer and plotter[9]. The instruments were calibrated with polyvinyl toluene latex beads of 1.305-μ mean diameter. Replicate 49.5-ml. samples of culture generating in the logarithmic phase were treated with penicillin, kanamycin, and rifampin stock solutions to achieve final concentrations of 24.0, 4.0, and 8.7 mcg./ml., respectively. Samples were taken every 25 min., and cell size distribution was obtained. A culture generated in the absence of drug was studied as a control.

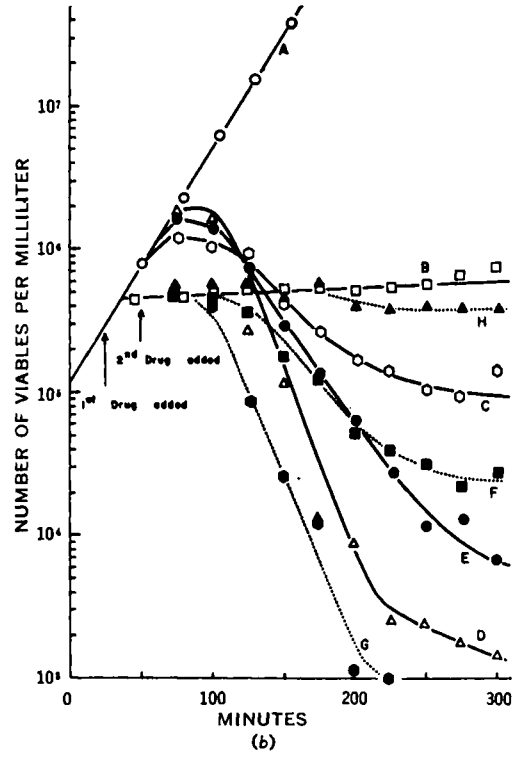
Bacterial Resistance to Penicillin Action—Aliquots (0.5 ml.) of sodium penicillin G solutions were added to cultures generating in the logarithmic phase to achieve final concentrations of 7.0 (Culture A), 9.0 (Culture B), and 12.5 (Culture C) mcg./ml. The drug-affected cultures of A, B, and C were allowed to generate for 15 hr. at 37.5° in an incubator. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium, brought up to the logarithmic growth phase, and diluted 100-fold again with fresh medium so that the original penicillin concentrations were diluted 10,000-fold. (See section on *Bacterial Culture.*) Aliquots of this solution were treated with various amounts of penicillin. The microorganisms generated from Culture A were treated with drug concentrations of 7.0, 10.0, 20.0, 39.6, and 77.7 mcg./ml.; those generated from Culture B were treated with 9.0, 20.0, 39.6, 77.7, and 114 mcg./ml.; and those generated from Culture C were treated with 12.5, 20.0, 39.6, 77.7, and 114 mcg./ml. Coulter counts were obtained every 25 min. for all drug-affected generations. The microorganisms generated from Culture C and treated with 12.5 mcg./ml. of sodium penicillin G were aseptically filtered 55 min. after the drug addition. The filtrate was inoculated with fresh organisms in the logarithmic generation phase.

A fresh broth was inoculated with the filtered organisms and treated with fresh drug solution to give a final concentration of 12.5 mcg./ml. of sodium penicillin G.

Bacterial Resistance to Kanamycin and Rifampin—Aliquots (0.5 ml.) of kanamycin and rifampin solutions were added to cul-

[9] Coulter Electronics, Inc.





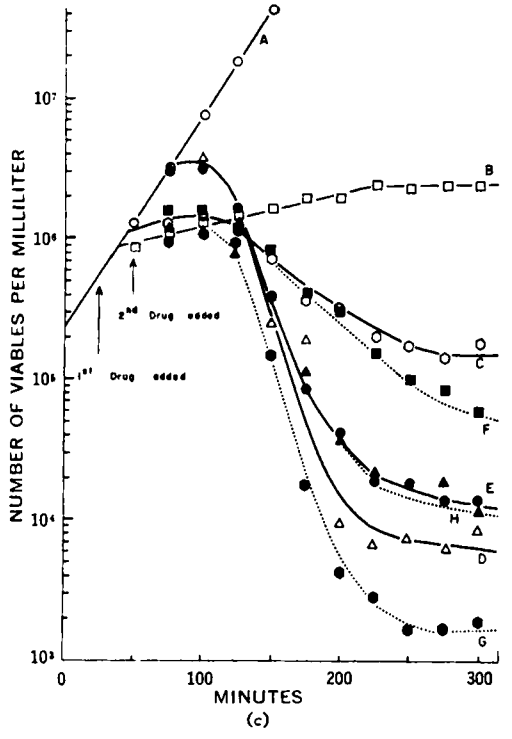


Figure 6—*Effects of combined action of penicillin, kanamycin, and rifampin with tetracycline* (a), *chloramphenicol* (b), *and novobiocin* (c) *on generation rates of* E. coli. *Curve A results from the generation of the culture in the absence of drug. Curve C results from the generation of the culture in the presence of 10.0 mcg./ml. of sodium penicillin G, curve D for 2.50 mcg./ml. of kanamycin sulfate, and curve E for 6.00 mcg./ml. of rifampin.* (a) *Curve B results from the generation of the culture in the presence of 0.30 mcg./ml. of tetracycline hydrochloride. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the tetracycline-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.* (b) *Curve B results from the generation of culture in the presence of 1.90 mcg./ml. of chloramphenicol. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the chloramphenicol-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.* (c) *Curve B results from the generation of culture in the presence of 90.0 mcg./ml. of novobiocin. Curve F results when sodium penicillin G (10.0 mcg./ml.) is added to the novobiocin-affected culture of curve B. Curve G results when kanamycin sulfate (2.50 mcg./ml.) is added to the culture of curve B, and curve H results when rifampin (6.00 mcg./ml.) is added to the culture of curve B.*

tures in the logarithmic growth phase to achieve final concentrations of 4.0 mcg./ml. of kanamycin and 8.7 mcg./ml. of rifampin. The drug-treated cultures were allowed to generate in an incubator for 15 hr. at 37.5°. An aliquot (0.5 ml.) was then diluted into 49.5 ml. of fresh culture medium, brought up to the logarithmic growth phase, and diluted 100-fold again so that the original drug concentrations were diluted 10,000-fold. (See section on *Bacterial Culture.*) Aliquots were treated with various amounts of the respective antibiotics.

The cultures of rifampin- and kanamycin-treated *E. coli* were again challenged with rifampin (curve A in Fig. 5) or kanamycin (curve B) to achieve final concentrations of 8.7 and 4.0 mcg./ml., respectively. One hundred fifteen minutes after rifampin addition and 145 min. after kanamycin addition, the cultures of curves A and B were filtered and the filtered organisms were allowed to generate in fresh broth medium. Aliquots of the cultures of the filtered organisms were diluted into fresh medium and treated with rifampin (curve C) or kanamycin (curve D) to give final concentrations of 8.7 and 4.0 mcg./ml., respectively. The filtrates of cultures of curves A and B were inoculated with aliquots of fresh *E. coli* generating in the logarithmic growth phase and gave curves E and F, respectively.

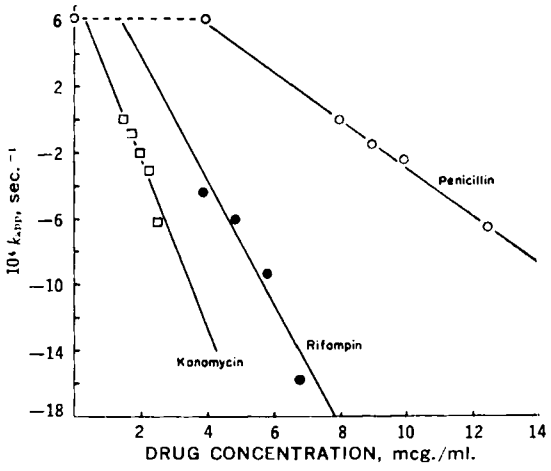


Figure 7—*Dependence of the apparent generation rate constant, k_{app}, for* E. coli *in Medium 3 USP on sodium penicillin G, kanamycin sulfate, and rifampin concentrations. The curves are constructed from two separate experiments.*

Effect of pH on Kanamycin-Affected Generation Rates—Six replicate 49.5-ml. volumes of medium, each buffered at a different pH value, were inoculated with an *E. coli* culture in the logarithmic generation phase. Drug solutions were added to the aliquots of these replicate cultures to achieve desired concentrations of kanamycin. Coulter counts were obtained for samples withdrawn every 25 min.

Potentiometric Determination of pKa Values of Kanamycin—Ninety-four milligrams of kanamycin sulfate was dissolved in 2.5 ml. of 0.2 *N* NaOH solution. A direct-reading pH meter[10] with a combination pH electrode[11] was used for pH measurements. The titrant was 1 *N* HCl solution and was delivered by a microburet[12] having a total capacity of 2 ml. and reading to 0.001 ml. on a microgauge. A magnetic stirrer kept the solution agitated. The procedure was repeated for a blank titration of a solution prepared like the sample solution with only the drug omitted.

Action of Penicillin, Kanamycin, and Rifampin in Combination with Bacteriostatic Antibiotics Tetracycline, Chloramphenicol, and Novobiocin—Replicate 49.5-ml. volumes of a culture containing 4×10^5 *E. coli*/ml. generating in the logarithmic phase (curve A in Fig. 6*a*) were treated with tetracycline (curve B), penicillin (curve C), kanamycin (curve D), and rifampin (curve E) to give final concentrations of 0.30 mcg./ml. of tetracycline hydrochloride, 10.0 mcg./ml. of sodium penicillin G, 2.50 mcg./ml. of kanamycin sulfate, and 6.00 mcg./ml. of rifampin, respectively. Twenty-five minutes after the addition of the first drug, replicate samples of the tetracycline-treated culture of curve B were treated with penicillin (curve F), kanamycin (curve G), or rifampin (curve H) to achieve final concentrations of 10.0 mcg./ml. of sodium penicillin G, 2.50 mcg./ml. of kanamycin sulfate, and 6.00 mcg./ml. of rifampin, respectively, with each containing the 0.30 mcg./ml. of tetracycline. Samples were taken every 25 min. Coulter counts were obtained for the drug-free culture (curve A) and tetracycline-treated culture (curve B), and viable counts were obtained for penicillin-, kanamycin-, or rifampin-treated cultures (curves C–H).

The experiment was reported for the combined action of penicillin, kanamycin, and rifampin with 1.90 mcg./ml. of chloramphenicol (Fig. 6*b*) and 90 mcg./ml. of novobiocin (Fig. 6*c*).

RESULTS AND DISCUSSION

Effects of Drugs on Microbial Generation—The numbers of *E. coli* as a function of time as affected by various concentrations of penicillin, kanamycin, and rifampin are plotted semilogarithmically in Figs. 1, 2, and 3, respectively. Certain general features of these curves are common to all three drugs. Subsequent to drug inoculation of the medium, the apparent generation rates of viables, as monitored by colony counts, decrease until the number of viables reach a maximum. These maxima are lower and occur faster with time the greater the drug concentration. Subsequent to these maxima and at the higher drug concentrations, the numbers of variables decrease and approach a first-order decay as manifested by a linear plot of the logarithm of viables against time. At the lowest concentration of drugs studied (7.0 mcg./ml. of sodium penicillin G, 1.5 mcg./ml. of kanamycin sulfate, and 3.88 mcg./ml. of rifampin), the generation rate was slowed until a relatively constant value of viables was achieved.

Subsequent to drug inoculation of the medium, the generation rate of total organisms, as monitored by Coulter counts, diminished and approached a constant value of plateau that persisted with time. These plateau values were lower with increasing concentrations of the drug.

The significant decreases in the numbers of *E. coli* per milliliter obtained by viable counts as a function of time compared to the total numbers obtained by the Coulter counter show that penicillin, kanamycin, and rifampin kill organisms at all of the studied concentrations (Figs. 1–3).

There were definite lag periods (Figs. 1–3) after drug addition before the slopes of the plots of logarithms of viable numbers against time decreased, and the effect was most pronounced with respect to kanamycin (Fig. 2). There were periods of slowed generation rates before the semilogarithmic plots decreased linearly with time. These intervals can be assigned to the time dependence of the drug partitioning process into the bacterial membrane, and their durations were apparent functions of drug concentrations.

The rate of loss of viables after these lag and induction periods may be expressed as the difference between the rate of generation and that of killing, where the latter is a function of drug concentration, *D* (1). If the kill rate is proportional to the first power of drug concentration:

$$dN/dt = (k_0 - k_D D)N \qquad \text{(Eq. 1}a\text{)}$$

$$dN/dt = k_{app}N \qquad \text{(Eq. 1}b\text{)}$$

where k_0 in the generation rate constant in the absence of drug, k_D is the kill rate constant, and N is the number of viable microorganisms. The apparent first-order generation rate constant, k_{app}, can be obtained from the slopes of the linear portions of semilogarithmic plots of number of viable counts against time in the drug-affected steady state prior to the appearance of possible resistant organisms, which may explain the terminal increase in microbial generation as manifested in Fig. 1.

The plot of k_{app} values against drug concentration demonstrates a linear dependence of k_{app} on drug concentration in accordance with the expectations of Eqs. 1*a* and 1*b* (Fig. 7). However, the actual function is:

$$k_{app} = k_0 - k_D(D - D^*) \qquad \text{(Eq. 2)}$$

A definite concentration of antibiotic must be exceeded before antibacterial activity is manifested. This minimum concentration, D^*, possibly may be assigned to the binding or removal of effective antibiotic concentration by the components of the medium (1). A maximum killing rate probably cannot be exceeded at very high concentrations of the antibiotics.

When the numbers of viables are negligible with respect to the numbers of dead organisms, the total numbers approach plateau values (Figs. 1–3). The plateau number, N_p, is some function of drug concentration and thus should be a function of the k_{app} value. Plots of k_{app} values against the logarithms of the ratio of the plateau number to the number at the time of drug addition, N_0, are shown in Fig. 8 for penicillin, kanamycin, and rifampin. Once the empirical relationship between $\log (N_p/N_0)$ and k_{app} is established, the k_{app} values can be estimated by Coulter counts only. This method has been applied to the studies of bacterial resistance to penicillin (Fig. 9) and of the effect of pH on kanamycin action (Fig. 10).

Effect of pH on Kanamycin Action—The k_{app} values may be estimated from the plateau numbers of total organisms from the correlations of Fig. 8. The estimated k_{app} values for cultures maintained at various pH values (6.30–7.50) are plotted against kanamycin sulfate concentrations in Fig. 10. The drug-free generation rate constants are independent of pH in this range (1), while the drug-affected apparent generation rate constants decrease with increasing pH at comparable drug concentrations. The slopes of the plots of Fig. 10 are representative of the killing constants, k_D, in Eq. 2 and

[10] Beckman Instruments, South Pasadena, Calif.
[11] Corning electrode, Sargent-Welch Scientific Co., Birmingham, Ala.
[12] Radiometer, Copenhagen, Denmark.

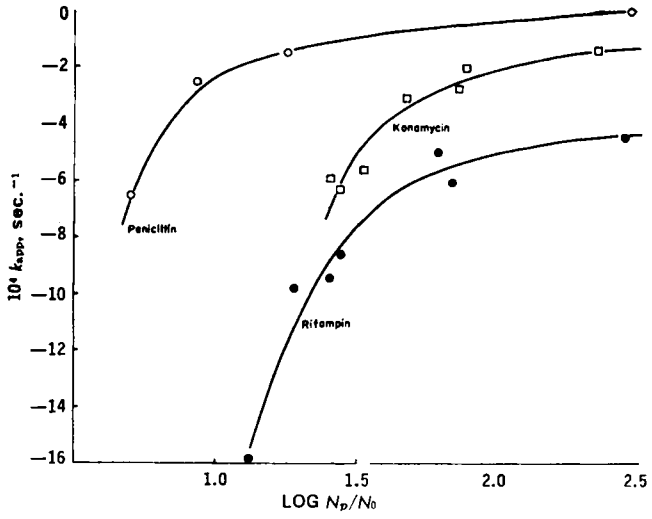


Figure 8—*Empirical relationship between the apparent generation rate constant, k_{app}, and the logarithm of the ratio of total counts at plateau (N_p) to the counts at the time of drug addition (N_0) for penicillin, kanamycin, and rifampin in Medium 3 USP.*

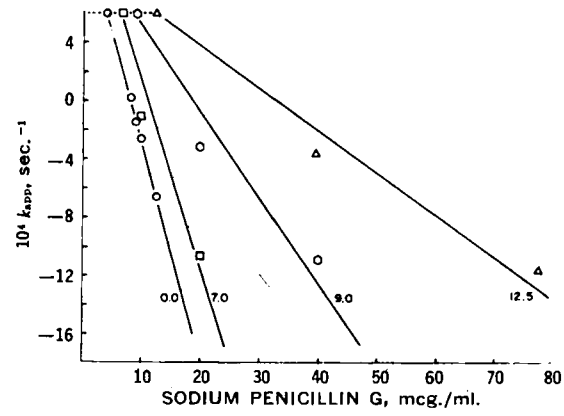


Figure 9—*Demonstration of the development of penicillin-resistant organisms on exposure to drug. Dependence of the apparent generation rate constant, k_{app}, for the generation of* E. coli *that has been exposed to penicillin on the sodium penicillin G concentration. The lines are labeled according to the sodium penicillin G concentrations, in micrograms per milliliter, to which the microorganisms were exposed for 15 hr. at 37.5° before these penicillin concentrations were diluted 10,000-fold and the concentrations of penicillin stated on the abscissa were added. The k_{app} values for viables were estimated from established correlations between rates of viable decay and plateau final values of total counts as given in Fig. 8.*

vary as a function of pH (Fig. 11). Potentiometric titration showed that kanamycin has four overlapping pKa values ranging from 6.5 to 9.7 (pKa_1 = 9.7, pKa_2 = 8.6, pKa_3 = 7.5, and pKa_4 = 6.5). The fact that the data in Fig. 12 are reasonably linear indicates that the less protonated or the unprotonated fraction of total kanamycin concentration is most probably the active species.

The minimum concentration, D^*, necessary for manifestation of antibacterial activity also decreases with increasing pH (Fig. 10) and implies that the inactivated species, possibly bound to components of the medium, is probably the more highly protonated.

Possible Development of Resistant Organisms to Drug Actions—Although there were indications from the terminal viable counts of the drug-affected organisms in the kanamycin and rifampin cases (Figs. 2 and 3) that some organisms remained that were resistant to the cidal action of these drugs but would not generate in their presence, it was clearly shown in the case of penicillin (Fig. 1) that there was either a population of organisms that did multiply in the presence of this drug or that the drug action had ceased after a time interval. This was manifested in the curves 200 min. after drug addition when the rate of decay of viables increased and eventually approached a typical first-order process of generation where the logarithmic number of viables was linear with time. The apparent parallelism of these terminal segments of the plots with that of a similar plot for *E. coli* generation in the absence of penicillin was indicative of negligible drug action.

Development of Penicillin-Resistant Organisms and Penicillin Inactivation—This phenomenon could be attributed to the consumption or degradation of the drugs, to the production of an inhibitor or inactivator of antibiotic action, and/or to the presence of bacteria able to resist the drug action. These bacteria could be present from the time of the microbial inoculation or could have arisen by mutation or adaptation processes occurring during the organisms' drug exposure. The cultures of *E. coli* were exposed to sodium penicillin G at concentrations of 7.0, 9.0, and 12.5 mcg./ml. for 15 hr., a time when the harvested organisms should be representative of a resistant strain if it exists (Fig. 1). When the original concentrations of penicillin were diluted 10,000-fold and the exposed organisms were challenged with the same concentrations of sodium penicillin G, they showed the same rates of generation as organisms in the absence of drug (Fig. 9). This is conclusive evidence that the subsequent increase in generation of viables of a culture exposed to penicillin was due to the presence of resistant organisms. The higher the drug concentration to which the organisms have been exposed, the greater is the drug concentration needed to kill organisms at the same rate (Fig. 9).

When the organisms that had been exposed to penicillin were filtered and used to inoculate fresh broth medium and were then challenged with penicillin, the generation rates of the filtered organisms were the same as the untreated *E. coli* in the absence of drug. When the filtrate was inoculated with naive organisms and challenged with penicillin, the generation rates were also the same as untreated *E. coli* in the absence of drug. These facts indicate that not only is the organism made resistant on exposure to penicillin but that the drug is degraded or inactivated in the culture. Thus, this drug degradation is a fast process and/or a penicillin antagonist is formed readily in the medium. The fact that penicillin is destroyed by β-lactamase produced by penicillin-resistant organisms (3) is consistent with this result.

The terminal data of the semilogarithmic plots of viables against time for various penicillin concentrations show an eventual increase in viables. The positive slopes of the plots of these terminal data appear to be parallel but have different intercepts (Fig. 1). Since the inoculum size for each study was the same, these penicillin-resistant organisms that manifest themselves are not the sole result of a small and constant fraction of the original inoculum that was penicillin resistant. Resistance must arise primarily during the time

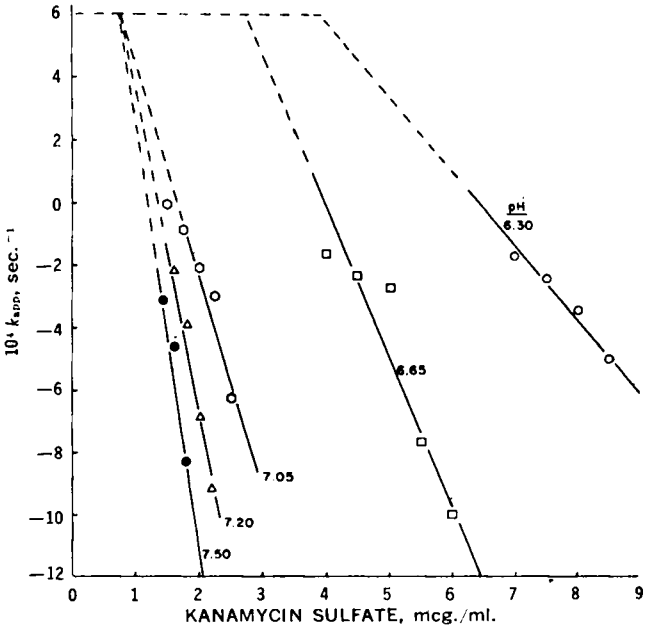


Figure 10—*Dependence of the apparent generation rate constant, k_{app}, for* E. coli *in Medium 3 USP on kanamycin sulfate concentration at various pH values.*

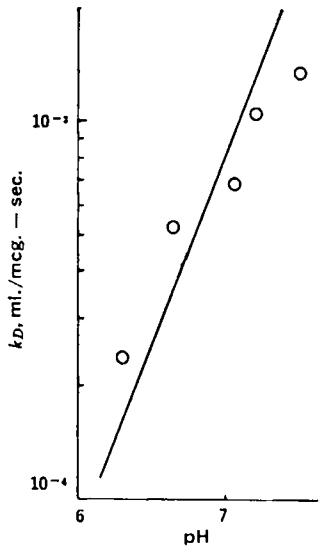


Figure 11—*Dependence of the killing constant, k_D, for the action of kanamycin on* E. coli *generation in Medium 3 USP on the pH value.*

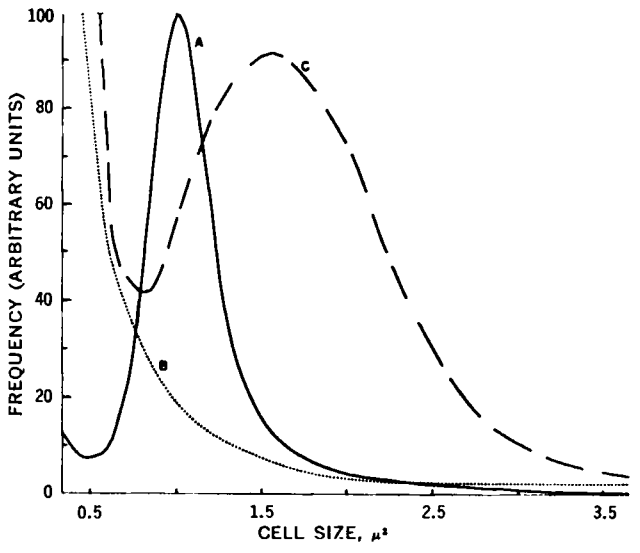


Figure 12—*Size-frequency distribution of the control organisms and (A) organisms treated with 4.0 mcg./ml. of kanamycin sulfate, (B) organisms treated with 24 mcg./ml. of sodium penicillin G 25 min. after drug addition, and (C) organisms treated with 8.7 mcg./ml. of rifampin 75 min. after drug addition.*

interval that the organisms are exposed to penicillin. The higher intercepts at lower penicillin concentrations imply the development of higher numbers of penicillin-resistant organisms at these concentrations for the same time after drug addition. Thus, the generation and production of resistants may be favored in the presence of lowered penicillin concentrations where some generation may still proceed.

Development of Rifampin- and Kanamycin-Resistant Organisms—When rifampin- and kanamycin-resistant organisms were filtered and the filtered organisms were tested in fresh medium against fresh drugs, the organisms were resistant to drug action (curves C and D in Fig. 5), whereas the filtrates showed drug activity when they were tested against fresh organisms (curves E and F). In these cases, therefore, the premise that the drugs are primarily destroyed or an antagonist is secreted can be ruled out, even though enzymatic drug inactivation was reported (4) to explain developed resistance to kanamycin and dihydrostreptomycin. This is consistent with the claim (5) that the resistance to rifampin is primarily due to the formation of a modified RNA polymerase insensitive to the action of rifampin. Analogously, streptomycin resistance was reported (6, 7) to be the result of modifications in the structure of the ribosomes that affect their affinity for streptomycin.

The studies of Fig. 4 clearly indicate the bactericidal action of kanamycin prior to the appearance of significant numbers of drug-resistant bacteria. The drug-affected bacteria did not generate significantly on dilution into media with lower drug concentrations or into fresh medium.

The size distribution studies (Fig. 12) show that kanamycin has no significant effect on cell size, whereas the cell size of rifampin-affected microorganisms gradually increases up to 75 min. after drug addition, at which time the cell size remains constant (curve C). In the case of *E. coli* killed by penicillin, no peak was apparent (curve B) shortly after drug addition. This result may be assigned to cells disintegrating into debris by the inhibition of cell wall synthesis so that cellular contents are dispersed and/or the cell membranes are devoid of contents and occupy lessened volumes. The fact that total counts (Fig. 1) achieved a plateau is indicative of this latter fact.

Combined Action of Penicillin, Kanamycin, and Rifampin with Tetracycline, Chloramphenicol, and Novobiocin—It has been claimed that various bacteriostatic drugs may interfere with the action of bactericidal antibiotics. The proposed explanation is that most bactericidal drugs only kill multiplying bacteria; when multiplication is inhibited by bacteriostatic drugs, killing cannot occur (8–11). For example, the early rate of bactericidal action of penicillin on several types of microorganisms was retarded by chloramphenicol *in vitro* (9–11) as well as *in vivo* (11–13) and by chlortetracycline or oxytetracycline *in vitro* (10, 14) and *in vivo* (13, 15). In many of these papers, the *number* of organisms killed by the bactericidal drug in the presence of the bacteriostatic eventually exceeded that in the presence of the bactericidal alone. Chloramphenicol, chlortetracycline, and oxytetracycline also interfered with the action of streptomycin (16) *in vitro* and in experimental infections of mice. However, reports of the combined action of the bacteriostatic sulfonamides and penicillin are conflicting; some state that these drugs are synergistic (17 26) and others that they are antagonistic (27–29). The conflicting reports may be due to differences in techniques, methods of measurement, and definitions of synergism and antagonism (29). If a bacteriostatic drug does interfere with the action of penicillin by inhibiting the multiplication of organisms, the stated cases of combinations with sulfonamides are difficult to reconcile with this premise.

An argument presented to rationalize an indifferent effect of sulfonamides on the initial action of penicillin is that sulfonamide has delayed action on microbial generation (30) and the penicillin activity may be exercised within that lag interval before the manifestation of sulfonamide inhibition of bacterial multiplication.

The premise that bacteriostatic drugs antagonize bactericidal drugs *in vitro* when the former are administered prior to or simultaneously with the latter has been based primarily on studies when the inocula of various organisms were in the stationary phase and were introduced at relatively large inoculum sizes (about 10^6–10^8 organisms/ml.) into the culture medium containing the drug combinations (9–11, 14, 16). The stationary phase occurred at 10^7–10^9 organisms/ml., so normally only a 10-fold increase in concentrations of organisms was possible. Thus, the actions of such drug combinations have been evaluated against microorganisms that were not in steady-state balanced generation, *i.e.*, the logarithmic phase, but were undoubtedly in the initial early lag phase where rates of generation are low and slowly accelerating and where the main process is an increase in size of the microorganisms.

Figure 6 shows that the prior addition of the bacteriostatics tetracycline and novobiocin do not interfere with the bactericidal actions of penicillin and kanamycin on balanced growth cultures in the logarithmic growth phase when the number of *E. coli* was 10^6/ml. for the addition of the latter antibiotic. The rates of decrease of viable counts of curves C and D are not significantly different from that of curves F and G, which contrast with the observations of Jawetz and coworkers (9–16) for large inocula of organisms introduced in the lag phase to a medium containing the antibacterial combination.

In most cases, the pretreatment of organisms with bacteriostatic antibiotics (curves F and G) has an advantageous effect on the more complete action of bactericidal drugs. This may be attributed to the fact that there is an interval between penicillin and kanamycin addition and their cidal action wherein microbial generation continues. In this interval of penicillin and kanamycin contact with rapidly generating organisms, mutants or adaptations resistant to these antibiotics may arise that are not possible with a prior action of the bacteriostatic drugs.

The prior addition of novobiocin had no significant effect on the

bactericidal action of rifampin in the present studies (Fig. 6c). However, when tetracycline or chloramphenicol was added before rifampin, the combined action of tetracycline (curve H in Fig. 6a) or chloramphenicol (curve H in Fig. 6b) with rifampin was dramatically less than that of rifampin alone (curve E).

The postulate that penicillin, kanamycin, or possibly other bactericidal agents will not be active on organisms in the stationary growth state (30) or in the early lag phase affected by bacteriostatic agents cannot be generalized automatically to bacteriostatically affected organisms from balanced cultures and in the logarithmic generation phase. The physiology of resting bacteria may be completely different than that of organisms whose generation is inhibited by bacteriostatic agents in the logarithmic generation phase. It appears that microbial kinetics affected by bactericidal agents alone and in combination with bacteriostatic and other bactericidal drugs need reevaluation using balanced cultures at lower inoculum sizes.

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Pattern of Phenylbutazone Degradation

D. V. C. AWANG▲, A. VINCENT, and F. MATSUI

Abstract □ Phenylbutazone and phenylbutazone–antacid formulations were examined by TLC for the presence of decomposition products. A procedure was developed for minimizing on-plate oxidation of phenylbutazone during TLC analysis, and preparative TLC was utilized for isolation of the major products of decomposition. Unequivocal identification of the major products of degradation was made by NMR and mass spectrometric determination of isolated material. Official phenylbutazone tablets underwent only trace oxidation whereas phenylbutazone–antacid preparations gave evidence of significant levels of oxidation and hydrolysis products. An accelerated decomposition study of the bulk drug and its products of degradation was also conducted.

Keyphrases □ Phenylbutazone and phenylbutazone–antacid formulations—isolation and identification of degradation products □ TLC—isolation, degradation products in phenylbutazone and phenylbutazone–antacid formulations

Two publications have appeared dealing with an evaluation of the integrity of phenylbutazone formulations: Beckstead *et al.* (1) examined both *official* and *alka*[1] preparations in the form of tablets and capsules, while Pawelczyk and Wachowiak (2) were concerned with aqueous injection solutions of sodium phenylbutazone and suppositories.

The former publication outlined an assay procedure for phenylbutazone employing acid–base extraction and determination by UV spectrophotometry. It also described a TLC system to facilitate identification and

[1] The term "alka" was used to designate preparations containing aluminum hydroxide and magnesium oxide or carbonate.